January 2015

INVESTIGATIONS INTO THE ANTI-COLLAGENASE EFFICACY OF SERUM AND PLASMA

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Graduate School Form 30
Updated 1/15/2015

PURDUE UNIVERSITY
GRADUATE SCHOOL
Thesis/Dissertation Acceptance

This is to certify that the thesis/dissertation prepared

By Emily Dakin Conway

Entitled

INVESTIGATIONS INTO THE ANTI-COLLAGENASE EFFICACY OF SERUM AND PLASMA

For the degree of Master of Science

Is approved by the final examining committee:

Jean Stiles
Chair

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Head of the Departmental Graduate Program Date

INVESTIGATIONS INTO THE ANTI-COLLAGENASE EFFICACY OF SERUM AND PLASMA

A Thesis

Submitted to the Faculty

of

Purdue University

by

Emily D Conway

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science

August 2015

Purdue University

West Lafayette, Indiana

This thesis is dedicated to my family for all their hard work and support that have enabled me to pursue my goals. I would not be here without them.

ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Lynn Guptill and Anisa Dunham for their technical assistance with this research; their help has been invaluable. I would also like to acknowledge the cats, dogs and horses that contributed to these projects.

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LIST OF ABBREVIATIONS

α1-PI : α1-Proteinase Inhibitor

API-PRP: Autologous Platelet Integrated-Platelet Rich Plasma

BLAST: Basic Local Alignment Search Tool

BSS: Balanced Salt Solution

EDTA: Disodium Ethylene Diamine Tetraacetate

EGF: Epithelial Growth Factor

E-PRP: Eye Platelet Rich Plasma

FFP: Fresh Frozen Plasma

FGF: Fibroblast Growth Factor

HP: Hydroxyproline

MMPs: Matrix Metalloproteinases

NAC: N-acetylcysteine

PDGF: Platelet Derived Growth Factor

PMNL: Polymorphonuclear Leukocytes

PRP: Platelet Rich Plasma

RFRP: Regenerative Factor Rich Plasma

TGF-β: Transforming Growth Factor β

TIMP: Tissue Inhibitor of Matrix Metalloproteinase

ABSTRACT

Conway, Emily D. M.S., Purdue University, August 2015. Investigations into the anti-collagenase efficacy of serum and plasma. Major Professor: Jean Stiles.

Corneal ulcers are a commonly encountered problem in domesticated species and can cause significant pain, incur a high cost for the owner, and result in blindness or eventual enucleation. The progressive deepening of corneal ulcers is mediated by enzymatic destruction of stromal collagen (termed keratomalacia) when collagenases from endogenous and exogenous sources are active on the corneal surface. The inhibition of collagenases can therefore be crucial to mitigating the damage that breakdown of corneal stromal collagen causes. In ulcers where collagenase activity is suspected, the use of topical serum has been recommended, in both human and veterinary medicine, primarily due to the presence of an endogenous serum anti-collagenase protein molecule: α-2 macroglobulin. Alpha-2 macroglobulin is produced by the liver and is able to inactivate collagenase molecules from various endogenous and exogenous sources. Plasma has also been recommended for use, in various forms, for the treatment of various corneal conditions. Plasma, in addition to containing α-2 macroglobulin, also contains platelets and other growth factors that may be useful in promoting corneal healing. Despite widespread recommendations for the topical use of serum to treat keratomalacia, there is little data on its storage and usage in domestic species. Additionally, there is little

information available comparing the anti-collagenase efficacy of serum to that of plasma. The goals of this research were to determine the effects of storage time and temperature on the anti-collagenase efficacy of serum in an in vitro corneal degradation model. This model uses a bacterial collagenase to cause collagen degradation, which is then measured by calculating percent corneal weight loss and quantifying the level of HP (a breakdown product of collagen) present; serum from different time and temperature storage is added to try to decrease weight loss and levels of HP. A reduction in percent corneal weight loss or HP would be indicative of a protective, anti-collagenase effect of the tested substance. An investigation into interspecies use of serum in the same model was also undertaken, to determine if any differences in anti-collagenase efficacy are seen with homologous serum compared to heterologous serum. In a second experiment, the anti-collagenase efficacy of fresh serum was compared to that of plasma for feline, canine and equine species. The hypothesis was that storage conditions would not affect the anti-collagenase efficacy of serum and that homologous and heterologous serum would have equal anti-collagenase efficacy. A final hypothesis was that fresh serum and plasma would have equal anti-collagenase efficacy. For the corneal degradation model, normal corneas from recently euthanized cats, dogs and horses were collected and stored at -80°C until use. For the first experiment serum was collected from healthy cats, dogs and horses and pooled by species, then stored for 30, 90 or 180 days at both -20°C and -80°C. Serum was pooled to control for any individual variation in serum composition. Sections of cornea were dried, weighed and incubated with clostridial collagenase and serum (homologous/heterologous) from each time/temperature point for the first experiment. Negative control samples were incubated only in saline with added calcium chloride

while positive controls contained saline, calcium chloride and clostridial collagenase. Corneal damage was assessed by percent corneal weight loss and HP concentration of the incubation fluid compared to positive and negative control samples. A Shapiro-Wilk statistic was used to test for normality on percent corneal weight loss and HP. If the distribution was normal, mean and standard deviation (SD) were reported, if the distribution was not normal then median and range were reported. If the data was normal, then a general linear model was used to compare the means across groups, otherwise the Kruskal-Wallis test was used. If the Kruskal-Wallis test or the general linear model was significant, then pairwise comparisons with Bonferroni adjustment. A P value of <0.05 was considered significant. Statistical software was used for all analyses. The inclusion of serum resulted in significantly less percent corneal weight loss compared to positive controls ($P<0.001$). Storage time ($P=0.074$) and temperature ($P=0.526$) did not affect percent corneal weight loss, while interspecies cornea/serum combinations (i.e. heterologous serum) ($P=0.028$) did affect percent corneal weight loss. The inclusion of feline or equine sera significantly reduced HP concentration ($P<0.001$) compared to positive controls. Significantly more HP was present in samples incubated with serum stored for 90 days compared to all other time points ($P<0.001$) while temperature did not significantly affect HP concentrations ($P=0.132$). For the second experiment, the ulcer degradation model used was the same as for the first experiment. However, in the second experiment, the sections of feline, canine and equine cornea were dried, weighed and incubated with clostridial collagenase and fresh serum or plasma. For the second experiment, blood was collected from healthy cats, dogs and horses; fresh serum or plasma was pooled by species and used in the model. Serum and plasma were pooled to

control for any individual variation in composition. To analyze the data from the second experiment, percent corneal weight loss and HP levels were compared among groups for positive control, serum and plasma samples with Kruskal-Wallis tests. If the Kruskal-Wallis test was significant (i.e., $P<0.05$), then Dunn's tests were performed for pairwise comparisons. A Spearman correlation coefficient was used to assess correlation between percent corneal weight loss and HP concentration. In the second experiment, both serum and plasma were significantly effective at reducing percent corneal weight loss in this model compared to positive control samples. No significant difference was found between feline ($P=0.579$), canine ($P=0.249$) or equine ($P=0.406$) corneas incubated with serum or plasma with regards to percent corneal weight loss. Canine serum and plasma significantly reduced HP levels while inclusion of feline and equine serum or plasma did not, compared to positive controls. Levels of HP were moderately correlated with percent corneal weight loss for feline sample ($P=0.002$), weakly correlated for equine samples ($P=0.096$) but were not correlated with percent corneal weight loss for canine samples ($P=0.842$). These studies confirm that both serum and plasma exert an anti-collagenase effect. The results of these studies suggest that serum can be stored for up to 6 months, at either -20°C or -80C°C without loss of anti-collagenase efficacy. The use of heterologous serum may be beneficial but further research is needed before any clinical recommendations can be made. Additionally this research indicates that plasma may be an acceptable substitute for serum as a topical corneal anti-collagenase treatment.

CHAPTER 1. INTRODUCTION

The cornea plays an integral role in vision, as it allows light to enter the eye, refracts that light and provides support and protection for intraocular contents. The epithelial surface of the cornea is composed of layers of non-keratinized squamous epithelial cells that cover the fibrous corneal stroma, the thickest layer of the cornea. Descemet's membrane lines the deep aspect of the corneal stroma, as the basement membrane for a single layer of corneal endothelial cells.[1] Although corneal epithelium is maintained by a constant cycle of growth of basal cells and sloughing of superficial cells, the corneal stroma is more static, with only a few active cells at any given time.[2]

Any defect in which the corneal epithelium is missing can be termed a corneal ulcer. Superficial corneal ulcers can heal via re-epithelialization in a matter of days. If however, a complication such as infection or an imbalance of normal corneal maintenance occurs, a simple corneal ulcer can extend into the stroma via enzymatic destruction of corneal stromal collagen, often referred to as corneal melting or keratomalacia.[3] These collagenase enzymes can be released by bacteria or fungal organisms that have colonized the ulcer, from neutrophils that invade the cornea secondary to the insult, or can be released from corneal cells themselves. Regardless of

the source of the enzyme, the end result is the same: degradation of stromal collagen and deepening of the ulceration. If allowed to progress unchecked, this destruction of stromal collagen can result in descemetoceles, corneal perforations, blindness and/or complete loss of the globe itself (enucleation).

In an effort to slow or arrest the loss of corneal stroma due to enzyme destruction, anti-collagenase substances have long been used, both topically and systemically, in human and veterinary medicine.[3-7] Anti-collagenases include tetracycline antibiotics, chelating agents and blood products (serum and plasma). Blood derivatives such as serum and plasma have some potential benefit over other topical anti-collagenases: they are relatively easily obtained, non-toxic and generally non-irritating. The use of topical serum is widely recommended in the veterinary and human literature as an adjunctive measure in the treatment of melting or deep ulcers.[3-7] Currently the ophthalmology department at the Purdue University Veterinary Teaching Hospital maintains a store of canine serum for topical use in canine and feline patients, as it can be difficult to obtain feline serum. If a large animal patient requires serum, it is generally obtained directly or from a healthy donor. Despite the widespread use of serum, there is a general lack of data regarding its usage in clinical practice.

Plasma has been investigated in human medicine for its use in treating a multitude of ocular disorders, including dry eye, chemical burns and non-healing ulcerations. Plasma, which can be purchased commercially, has potential benefits as a topical therapy over serum in that it is available commercially, ensuring a steady source for clinical

usage. To the knowledge of the author, few investigations have been undertaken to quantify the efficacy of topical canine plasma as an anti-collagenase in veterinary medicine, and there has been no investigation of the anti-collagenase efficacy of feline and equine plasma.[8,9]

The corneal degradation model used here was based off of previous work with equine corneas, where dehydrated corneas were incubated in solution containing bacterial collagenase and various anti-collagenases.[10] The degradation model used percent corneal weight loss and concentration of HP(a breakdown product of collagen) to quantify collagenase induced degradation of corneal collagen.[10] A reduction in corneal weight loss and/or HP concentration would indicate efficacy of the tested anti-collagenase. The research performed for this thesis assessed the effects of storage time and storage temperature on the in vitro anti-collagenase efficacy of feline, canine and equine serum in a corneal degradation model. Interspecies (heterologous) use of serum was also investigated. A secondary portion of this research project compared the anti-collagenase efficacy of fresh serum to that of fresh plasma in the same corneal degradation model in feline, canine and equine species.

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CHAPTER 2. LITERATURE REVIEW

2.1 Corneal Anatomy

The globe is composed of 3 tunics: an inner neurosensory layer, a vascular layer and an outer fibrous layer.[1] The cornea, the clear anterior portion of the fibrous tunic of the eye, plays a vital role in vision as it both provides protection of intraocular contents and also allows the passage and refraction of light into the eye.[2] The cornea is itself protected by the upper and lower eyelids and the nictitating membrane; it receives its nourishment from the pre-corneal tear film and from the aqueous humor that fills the anterior chamber.[1]

Domesticated species have 4 distinct layers to the cornea, while humans and non-human primates have 5 layers.[3] The layers of the cornea, from anterior to posterior are the corneal epithelium, Bowman's layer (human/non-human primate), corneal stroma, Descemet's membrane and the corneal endothelium.[1-4]

The corneal epithelium provides protection for the cornea and also helps to provide a smooth outer surface for the refraction of light.[5] The corneal epithelial thickness varies among species, being slightly thicker in domestic ungulates compared to domestic carnivores, such as the dog and cat.[1] A single layer of columnar cells makes up

the basal layer of the corneal epithelium. The basal cells are attached to the underlying basement membrane via hemidesmosomes.[2] These cells are continuously replicating and as they do so, the cells move closer to the surface of the eye and begin to flatten out and become polygonal in shape, at which point they are called 'wing cells'.[3] The number of wing cells varies among species, with dogs and cats having 2-3 layers of these cells.[1] As these cells are pushed anteriorly, they become more flat and become non-keratinized squamous epithelial cells. The basal cells, wing cells and squamous cells are attached to one another via desmosomes which are present in greater numbers in superficial layers; gap junctions are also found in all layers but are more numerous among the deeper layers.[2-4] Tight junctions also exist among the most superficial layers of epithelial cells, so that a semi-permeable membrane is formed.[2] These tight junctions help to prevent the infection of the corneal stroma by organisms such as bacteria and fungi.[5] These superficial epithelial cells are subsequently shed, to be replaced by younger cells beneath them; the entire epithelium can be replaced in 7 days.[2,4,5]

Briefly, Bowman's layer is an acellular membrane located beneath the corneal epithelium but anterior to the corneal stroma; as previously mentioned, this layer is not present in domesticated species.[1,3] When it is present, it is approximately 12 μm thick and is located beneath the epithelial basement membrane.[2] Bowman's layer is comprised of fibrils of collagen and proteoglycans; the function of this layer is unclear.[4]

The corneal stroma is the largest layer of the cornea, comprising approximately 90% of the overall corneal thickness.[1-4] The majority of the dry weight of the corneal

stroma is collagen (which accounts for 70-82% of the dry corneal weight).[6,7] The corneal stroma is composed of collagen fibrils, keratocytes and an extracellular matrix of proteoglycans and glycoproteins.[4] The collagen fibrils are uniform in both size and in orientation; corneal collagen fibrils are arranged in parallel in numerous lamellae. Most human collagen fibrils are 27-35 nm in diameter, although there are a few that measure up to 70 nm in diameter.[2,3,8] This uniformity allows light to pass relatively unimpeded through the cornea. The corneal stroma contains multiple types of collagen, which are differentiated from one another by the composition of their genetically determined polypeptide chains.[9,10] In the cornea, collagen Type I is most common and it forms stromal fibrils; these fibrils also contain collagen type V, thought to be associated with the uniformity and growth of the stromal collagen fibril.[1,2,7,8,11-14] The majority of the stromal fibril (80-94%) is collagen type I, the remainder is collagen type V.[13,15] Collagen type VI is only present around the collagen fibrils and is involved in the stability of the stromal matrix and organization of the stromal fibrils.[1,8,11,13,14] Collagen type III is thought to be related to corneal development, as it is present in fetal corneas but only in very low levels in adult corneas.[1,7,11,16] Collagen type III is postulated to provide a framework for the deposition of collagen type I during development; this may also explain why it is present in corneal scars.[16,17] Basement membranes of the cornea also have specific types of collagen present; collagen type VII is located in corneal epithelium and collagen type VIII is found in Descemet's membrane.[11] A human study using immunofluorescence detected collagen type I in the corneal stroma and in corneal scars, collagen type III in corneal scars, and collagen type IV in both the epithelial basement membrane and Descemet's membrane.[17]

The corneal stroma also consists of an extracellular matrix made of glycoproteins and proteoglycans such as keratin sulfate and dermatan sulfate.[1-3,8] The most abundant type of proteoglycan in the extracellular matrix is keratin sulfate.[18] The extracellular matrix is located around the fibrils of collagen and is produced in part by the various cells of the cornea, including epithelial cells and stromal keratocytes.[11,18,19] The keratocytes present within the corneal stroma help to maintain the corneal lamellae; these cells comprise only about 10% of the stroma.[2,3] When activated after corneal wounding, keratocytes are able to lay down new collagen fibrils and extracellular matrix, although they cannot do so in the same uniform manner, resulting in a corneal opacity (scar).

Descemet's membrane is a very thin, acellular membrane located just posterior to the deep corneal stroma. This layer, which thickens slightly with age, is the basement membrane of the corneal endothelium.[2,3] Descemet's membrane, which in the adult human eye is approximately 15 μm thick, is resistant to degradation by enzymes.[2]

The final layer of the cornea, the corneal endothelium, is a single layer of cuboidal epithelial cells that lines the inside of the cornea.[4] The endothelial cells themselves are polygonal cells, with most of the cells being hexagonal in shape, and are connected by both gap junctions and tight junctions.[2] The corneal endothelium serves an important role in maintaining the relatively dehydrated state of the cornea, which also contributes to corneal clarity. Sodium/potassium ATPase pumps in the endothelial cells help to remove excess fluid from the corneal stroma, allowing it to remain transparent.[1-3] The endothelial cells do not directly remove fluid from the corneal stroma; rather, they

move ions and create an osmotic gradient which moves water from the stroma to the anterior chamber.[4] However, the number of endothelial cells decreases with age and loss of these cells is permanent.[4]

Although turnover of the cornea is slow compared to other tissues, the cornea does have a system for routine maintenance, which is accomplished by a balanced system of removal of damaged tissue and replacement with healthy tissue.[18] This maintenance of corneal cells is managed by proteinases, which are enzymes that are able to breakdown proteins. There are four classes of endogenous proteinases: serine proteases, cysteine/thiol proteases, metalloproteinases and aspartic proteases.[20] In the cornea, this maintenance is mainly carried out by MMPs; a group of enzymes which affect the proteins of the stroma and extracellular matrix.[21] The MMPs are enzymes that are released as inactive pro-enzymes, which require zinc for activation.[21] MMPs can be further characterized by the protein molecules on which they act: collagenases (which break down collagen types I-III), stromelysins (which break down proteoglycans and other collagens), gelatinases (which break down denatured collagens/gelatin).[21-23] TIMPS are specific endogenous proteins which regulate the deposition of and breakdown of the extracellular matrix.[21,24,25] A non-specific inhibitor of MMPs, α2-macroglobulin, is found in serum.[26] A further discussion of MMPs and their role in corneal ulceration and repair is included in the next section.

2.2 Corneal Ulceration and Healing

Corneal ulceration is known to be a frequent cause of vision loss in humans and is a common diagnosis in veterinary medicine. A corneal ulceration can be loosely defined as any lesion of the cornea where the corneal epithelium is no longer present, with resultant exposure of the corneal stroma. A simple corneal ulceration can be described as a loss of corneal epithelium, without the presence of infection, which typically epithelializes within 5-7 days. The healing of the corneal epithelium has 3 important phases: cell migration, proliferation and adhesion.[27] When the corneal epithelium sustains an injury, there is a lag phase where the epithelial cells at the edge of the ulcer will lose their attachments to the underlying basement membrane, enlarge and slide to cover the exposed basement membrane.[5,28] The movement of cells to cover the defect begins within hours after the insult occurs and continues in a centripetal manner until the new epithelial layer is complete.[5,27] Once the defect is covered, continued division of basal cells restores normal corneal epithelial thickness.[1,5,27] As epithelial cells move to cover the stroma, they form short term attachments to specialized 'adhesion plaques' which are part of the extracellular matrix and are receptors for various parts of the basement membrane.[27] Hemidesmosomes are then formed by the basal epithelial cell layer to adhere to the basement membrane.[5,27,28] As long as the epithelial basement membrane is intact, the epithelial cells can adhere to it strongly in a matter of days, with the epithelium becoming functionally normal.[8] If a very large area of corneal epithelium is lost, new basal cells are supplied by stem cells at the corneal limbus, where the cornea and sclera meet; larger epithelial defects take longer to heal.[1,5,8,29] In cases where the

basement membrane has been damaged, it can take longer for the epithelium to develop a firm attachment, as epithelial cells must secrete new basement membrane.[8,27,28]

A complicated ulcer then can be described as an ulceration that has extended into the corneal stroma, often due to infection, which would not be expected to heal within 5-7 days. As soon as a few hours after stromal injury, PMNLs will arrive at the site of injury from the pre-corneal tear film, followed by activation of stromal keratocytes.[5,28,29] Keratocytes play an important role in corneal wound healing, as they will either undergo cell death or become activated into cells that can repair the damaged stroma.[30] A short time after the corneal epithelium is lost, the keratocytes directly beneath the area become apoptotic; this is thought to be due to the release of interleukin-1 from the remaining epithelial cells.[30] If the basement membrane of the epithelium is damaged, some of the keratocytes become activated, assuming many characteristics of fibroblasts, and move towards the damaged, acellular area.[30,31]

Within days of injury, the activated keratocytes begin to secrete collagen, proteoglycans and glycosaminoglycans; this new collagen is larger in diameter and is laid down in a disorganized manner.[24,29] Keratocytes are also able to transform into a myofibroblast type of cell, which assist with the contraction of the wound in addition to contributing to the extracellular matrix.[30] Fini used a rabbit penetrating keratectomy model to investigate corneal repair.[18] A fibrin plug was allowed to fill the defect, this plug was epithelialized within 48 hours. At 24 hours, activated keratocytes had moved

into the fibrin plug and by 2 weeks post injury, the defect was primarily repaired stroma.[18]

As the cornea heals, the ratio of collagen proteins changes. In one study, the ratio of collagen type I to collagen type V decreased, as type V collagen increased during the healing phase.[31] In human cadaver corneas from keratoconus patients treated with a corneal graft, collagen type III was detected by immunofluorescence in the healed cornea and in the corneal scars.[17] Wound strength increases as the amount of new collagen deposited increases, and continues to stabilize over a period of weeks to months.[28,29]

Several research projects have investigated the ability of the rabbit cornea to heal after wounding.[32,33] In a penetrating keratectomy model, Cintron found that the amount of DNA present increased during healing compared to normal tissue, then decreased over time until it reached normal levels, 10-13 weeks after the initial wound.[32] Over a similar time period, the amount of collagen present also increased, reaching more normal levels by approximately 9 weeks post injury. Extracellular matrix proteins not normally found in adult cornea were also detected. Taken together, these findings were interpreted as regeneration of the corneal stroma. Interestingly, despite collagen levels becoming more like normal cornea over a period of weeks to months; corneal transparency only returned to a near-normal state 1.5 years after the keratectomy.[32]

In 1978, Cintron examined the structural changes to rabbit corneas after penetrating keratectomy, finding that identifiable corneal lamellae were not present in the

scar tissue 3 weeks after the surgery; rather the fibrils were irregular in both orientation and size.[33] Even at over a year post injury, the fibrils present in the corneal scar were arranged differently to that of normal stroma.[33]

As previously mentioned, proteinases are important for corneal maintenance, wound healing and include MMP-1 (collagenase 1), MMP-2 (65 kD gelatinase/gelatinase A), MMP-3 (stromelysin 1), MMP-7 (72 kD gelatinase), MMP-9 (92 kD gelatinase/gelatinase B) and MMP-13 (collagenase 3). Stomelysins (including MMP-3) are able to break down proteoglycans, while collagenases (including MMP-1) break down collagen types I, II and III. [23,34] Gelatinases (MMP-2, MMP-9) degrade collagen types IV, V and VII and can also break down denatured collagen.[23,34] TIMP-1 and TIMP-2 are both also involved in corneal wound healing.[21] Both MMP-2 and MMP-9 are important with regard to stromal collagen. Both MMP-2 and MMP-9 can be produced from corneal epithelial and stromal cells and research has shown their levels increase after surgical keratectomy.[35] It has been suggested that MMP-2 is active both in maintenance of the normal cornea and in remodeling of the stroma after corneal injury.[35-37] In contrast, MMP-9 is not secreted unless corneal damage occurs; after corneal injury epithelial cells, keratocytes and polymorphonucleocytes can produce it.[12,22,35,36] It has been suggested that MMP-9 may assist in remodeling the epithelial basement membrane and enabling re-epithelialization.[12,22,35,36] In an experimental study, MMP-9 was present early after corneal wounding but was no longer detectable within 4 weeks of the original insult.[12] Derangements in the regulation of MMPs have been implicated in many disease processes, including neoplastic and cardiovascular disorders .[38]

Analysis of levels of MMP's in tears has been investigated with regard to corneal ulcerations in veterinary species. The activity of MMP-2 and MMP-9 were evaluated in the tears of 10 horses with ulcerative keratitis over the course of their disease.[39] Total tear MMP activity was significantly higher in ulcerated eyes compared to the normal eye and the mean activity of the MMPs decreased between hospital admissions and healing of the ulcer.[39] In dogs, tear samples were collected from canine patients with *Pseudomonas aeruginosa* associated keratitis and concentrations of MMP-2 and MMP-9 were assessed.[40] The levels of both latent and active MMP-2 and MMP-9 were higher in affected eyes compared to non-affected contralateral eyes; levels of both were also significantly less once affected eyes healed compared to when they were first evaluated.[40]

In human medicine, the pre-corneal tear film has also been evaluated with regards to healthy and ulcerated corneas. Prause examined the concentrations of PMNL neutral collagenolytic protease in the tears of 53 healthy persons and from 18 eyes of 16 people with melting corneal ulcers.[41,42] The protease was not found in the tears of any of the healthy patients but was found in the tears of those patients with melting corneal ulcers. Prause was also able to show a decrease in the amount of the protease in the tear film as the corneal ulcers healed.[42]

Finally, cytokines are also important for the healing of corneal ulcerations. EGF, FGF, TGF-β, PDGF, substance P and others, arising from the pre-corneal tear film, corneal nerves and epithelial and stromal cells, have been found to have beneficial effects on corneal healing.[28]

2.3 Collagenases

Corneal ulcers can occur for a myriad of reasons, including trauma, infection, chemical or thermal burns, nutritional deficiency, autoimmune diseases and others.[23,43,44] Despite the many possible causes of ulceration, one fact remains constant: the destruction of collagen allows for ulceration to progress. Ulcers that have extended into the corneal stroma are mediated by uncontrolled enzymatic breakdown of stromal collagen. This unregulated breakdown of corneal collagen is clinically termed keratomalacia or corneal melting. Collagenases are enzymes that have the ability to break down collagen; in vivo, collagenases have been found in eyes which have sustained chemical or thermal burns.[8] Although collagenases can also be produced by infectious agents, endogenous collagenases from white blood cells, corneal epithelial cells or stromal keratocytes/fibroblasts themselves have been implicated in the progression of stromal loss.[12,28,35-37,45] MMP-1 and MMP-2 and MMP-9 can be produced by corneal epithelial cells and keratocytes after corneal injury, although most MMP-9 is produced by epithelial cells.[46] In 1969, investigation of bovine cornea revealed peptidase activity and a collagenase capable of breaking down stromal collagen was identified from rabbit corneal epithelium.[47,48] Corneal collagenase isolated from rabbits has been found to be functional at physiologic pH and it has been documented to cleave collagen fibrils into ¾ and ¼ lengths, which is a characteristic of mammalian collagenases.[49,50] Human corneal collagenase has been found to be a true collagenase.[51]

Other sources of collagenase, including PMNLs, bacteria and fungi have been investigated as well. An in vivo study was undertaken to determine if rabbit corneal

collagen could be broken down by intra-stromal injections of active (lysed) PMNLs.[52] Injection of lysed PMNLs resulted in active corneal collagenolysis while the injection of heat inactivated PMNLs did not cause collagenolysis.[52] A later study also investigated the effects of PMNL in stromal ulceration, where alkali burned rabbit corneas were treated with glued on contact lenses in an attempt to prevent stromal ulceration.[53] In this study, the presence of a glued on methyl methacrylate lens prevented both infiltration of PMNL and epithelialization of the ulcer; it also prevented ulceration of the stroma itself. In rabbits where the lens was removed and epithelialization of the ulcer was allowed to take place, infiltration by PMNL and stromal ulceration followed. Thus Kenyon et al. postulated that growth of the corneal epithelium could encourage the infiltration of stromal PMNL, which resulted in further breakdown of corneal stroma.[53] In rabbit corneas that were already infiltrated with PMNL, prevention of epithelialization by either mechanical debridement or placement of a methyl methacrylate ring was not enough to prevent progressive ulceration of the corneal stroma. This demonstrated that PMNL were able to cause corneal melting on their own.[53]

Bacterial infection of the cornea can contribute to corneal collagen breakdown, through the release of proteolytic enzymes and also by stimulating an immune response where PMNL influx can result in collagenolysis.[54] The bacterium *Pseudomonas aeruginosa* is known to be particularly destructive with regards to corneal melting. Brown identified an enzyme from 5 different strains of *Pseudomonas aeruginosa* that was able to degrade the proteoglycan matrix of rabbit corneas after intra-stromal injection, despite the fact that this enzyme was not able to degrade the collagen fibrils

present.[55] These findings were corroborated by a later investigation where rabbit corneas were experimentally infected with *Pseudomonas aeruginosa*.[56] Histological sections of affected corneas showed loss of proteoglycans but normal collagen fibrils. This was in contrast to rabbit corneas treated with a collagenase from *Clostridium histolyticum*, where histologic examination showed damaged corneal collagen fibrils.[56] Another investigation into the effect of *Pseudomonas aeruginosa* on rabbit corneas determined that a combination of the protease produced by the bacterium and endogenous collagenases released by the host were responsible for corneal melting.[57]

Fungal organisms have also been identified as sources of enzymes capable of degrading stromal collagen. A clinical strain of *Aspergillus flavus* was isolated from a human case of severe fungal keratitis; the organism was able to produce 3 different types of proteases (metalloproteinase, serine and cysteine), all of which were able to degrade collagen in vitro.[58]

Regardless of the source of these destructive enzymes, they contribute to the breakdown of the corneal stroma. Continued loss of the stroma can result in devastating consequences for the patient, including loss of vision and/or the globe itself. As such, blockage of collagenase enzymes can be significant in mitigating further damage to the globe

2.4 Corneal Anti-Collagenases

Many agents have been recommended, in conjunction with other therapies (such as antibiotics and antifungal agents), to slow or stop the enzymatic destruction of stromal collagen in order to reduce the potential complications of corneal ulcers.[44,59-62] Anti-collagenase agents that have been investigated in relation to corneal disease include tetracycline antibiotics, NAC, EDTA, cysteine, ilomostat, tetanus antitoxin and others.

As has been previously noted, many collagenases are secreted as inactive and they require calcium and/or zinc to become functional.[21,49] As such, use of chelators such as EDTA and NAC have been recommended.[8,45,59] Both sodium and calcium EDTA are able to inhibit collagenase in vitro and have been recommended for use in veterinary patients as topical anti-collagenases.[44,49,62] Another chelator, NAC, has also been investigated and recommended for use.[44,62] The inhibitory effect of sodium and calcium EDTA and NAC were investigated and found to be maximally inhibitory in vitro at concentration of 10^2 molar.[49] The effect of NAC use was examined on rabbit eyes in vivo and was found to be irritating at concentrations of 20%.[63] Berman refutes this from personal experience, where rabbits treated with topical NAC did not show any adverse effects.[59] Since both EDTA and NAC are not able to irreversibly bind calcium (although NAC is less susceptible to reversibility), their anti-collagenase efficacy is reversible.[49,55,62] This means that they must be used frequently in order to continue to be effective, although it was postulated NAC would be the better anti-collagenase based on their finding that it's anti-collagenase effect was better conserved in vitro compared to EDTA.[49]

Tetracycline antibiotic have been recommended for use both systemically and topically in many ophthalmic disorders: dry eye, blepharitis and corneal ulceration.[64] Their use has been recommended as they possess anti-collagenase activity that is unrelated to their antibiotic activity.[64-66] An in vitro experiment tested minocycline, doxycycline and tetracycline on collagenase from rats' PMNL and found that all 3 substances could inhibit the collagenase by approximately 90%, compared to 0% inhibition by ampicillin and cefazolin.[66] The use of tetracycline antibiotics has been recommended clinically in the treatment of melting corneal ulcers.[44,60]

Multiple anti-collagenase agents were investigated with regards to their ability to inhibit MMP-2 and MMP-9 from the tear film of horses suffering from ulcerative keratitis.[67] Doxycycline, EDTA, NAC, Ilomostat, α1-PI and fresh equine serum were tested in vitro on tear samples obtained from ulcerated equine eyes. The total MMP activity was reduced by 96.3 % (doxycycline), 99.4% (EDTA), 98.8 %(NAC), 98.9% (ilomostat), 90% (equine serum), 52.4 (0.1% α1-PI) and 93.6% (0.5% a1-PI) when compared to the MMP activity of untreated equine tears.[67]

Synthetic collagenase inhibitors have also been investigated for anti-collagenase efficacy. Galardin (ilomostat) is a modified dipeptide which was investigated against a *Pseudomonas aeruginosa* protease in vitro and in vivo. Galardin was effective in vitro and was able to slow the onset of corneal melting in vivo in rabbits, although it was ultimately unable to prevent corneal melting.[68] A synthetic thiol based peptide was tested on rabbit corneas which had been experimentally infected with *Pseudomonas*

aeruginosa.[43] Compared to affected eyes treated with gentamicin or the negative control vehicle, treatment with the peptide was able to significantly delay corneal melting.[43]

Other therapies have been utilized to prevent corneal melting, with varying degrees of success. A study by Haffner et al. compared the anti-collagenase efficacy of tetanus antitoxin against that of equine serum and NAC in an in vitro model.[69] It was found that all 3 substances were able to decrease corneal collagen breakdown by 50% compared to positive control samples.[69] Corneal cross linking, a procedure where riboflavin is applied to the cornea and exposed to ultraviolet light leading to the development of new bonds among the corneal stoma, has been reported to be effective at arresting corneal melting in horses, dogs and cats.[70-71] This therapy however, requires specialized equipment to perform and is not widely available. *Aloe vera* was recently tested in vitro as an anti-collagenase. In this particular experiment, aloe solution was not able to block collagenase mediated destruction of collagen type I or gelatin; furthermore it actually increased the degradation of collagen type IV by the same enzyme.[72] One final recommended anti-collagenase is serum, which will be discussed in depth in the next section.

2.5 Serum

The Book of Dede Korkut, a book of Turkish stories from five centuries ago, tells of a blind woman who regains her sight after rubbing her eyes with blood; it has been postulated that this text describes the first ever use of serum as an ocular treatment.[73] The topical use of serum was recommended initially due to anecdotal evidence that corneas

that were vascularized did not ulcerate and the discovery that human serum contains collagenase inhibitors.[59,74] In a set of experiments investigating the anti-collagenase efficacy of serum, it was discovered that α1-antitrypsin was capable of inhibiting rabbit corneal collagenase although it did not inhibit human corneal collagenase; this protein was also not inhibitory against human collagenase in another investigation.[74,75] Alpha-2 macroglobulin was found to inhibit both human and rabbit collagenase.[74] A later study determined that the α-2 macroglobulin found in human and rabbit serum was able to exert its anti-collagenase action by forming complexes with the corneal collagenase.[76]

The serum protein α2-macroglobulin is produced by the liver; in humans it comprises 8-10% of serum proteins.[77] It is a 725 kilo Dalton glycoprotein comprised of 4 large sub-units.[26,78] The primary function of α-2 macroglobulin is to control any excessive activity of proteinases that are present in the body; α-2 macroglobulin is a significant inhibitor of collagenase; more than 90% of the anti-collagenase efficacy of serum is due to α-2 macroglobulin.[26,75,78,79] It is important to note that α-2 macroglobulin is a non-specific proteinase inhibitor, which means it is able to bind enzymes from all 4 classes of proteinases: serine, cysteine, aspartic and metalloproteinases.[78] Each α-2 macroglobulin is able to tightly bind collagenase molecules, rendering them inactive.[80,81] However, Berman notes that the large size of α-2 macroglobulin means that it is unlikely to become extravasated, which limits its natural utility, even when ulcerating corneas become vascularized.[45] This potential setback could be overcome by using serum topically, directly applying α-2 macroglobulin to the corneal ulcer.

Topical usage of autologous serum is widely recommended in the human and veterinary literature to treat a variety of ocular conditions.[44,59-62,82-84] In the human literature, serum has been recommended to treat dry eye, persistent epithelial defects and recurrent corneal erosions, neurotrophic keratopathy, superior limbic keratoconjunctivitis, aniridic keratopathy and Mooren's ulcers.[84] Despite this, in the human literature, although there are various recommendations for the production, use and storage of serum for topical usage, there is little clinical data regarding such claims. One report details the production and storage of serum for human ocular use at two different locations.[82] At the University of Lubeck in Germany, autologous serum is to be stored at 4°C for no greater than 16 hours or can be stored for up to 3 months if frozen at -20°C.[82] The National Blood Service in Wales and England stores blood at 4°C for 2 days; once obtained, the serum is frozen at less than -30°C and can be stored for up to 6 months, but should be discarded after 1 day of use.[82] No data are given to support these recommendations. In 2005, a report was published detailing an optimized protocol for the production of autologous serum eye drops.[83] This study examined the effects of preparation method on several factors contained in serum (fibronectin, growth factors and vitamins) and tested their ability to stimulate cell growth in a cell culture model.[83] They recommend allowing the blood to clot for longer than 120 minutes, centrifuging at 3000 g for 15 minutes followed by dilution of the serum with balanced salt solution. They then recommend storing the serum at – 20°C for up to 3 months; once in use bottles are stored at 4°C and discarded after 16 hours.[83] No data is given in support of the storage recommendations, only with regards to the collection of the serum. Tsubota et al. analyzed the effects of storage time on the concentration of EGF, TGF-β and vitamin A in human serum, which

was stored for one month at 4°C and 3 months at -20°C.[85] The results of this study showed that the concentrations of the measured components did not change significantly over the storage time periods; no investigation into the storage effect of α-2 macroglobulin was undertaken.[85]

In veterinary medicine, there also exist many recommendations for the use of topical serum in melting ulcers. Again however, there is little published data to support the claims made for the use and storage of serum for ocular use. One report notes that serum can be stored indefinitely if frozen, but it does not provide a specific temperature.[62] A single abstract investigated the effects of storage time and temperature on the anti-collagenase efficacy of serum using an in vitro model. In this model, equine serum was stored for 7 days at – 18C°C, 4°C and 23°C and the ability of the serum to inhibit MMPs from equine tears was measured daily.[a] There was no difference in inhibitory activity of the serum from the various storage temperatures and no decrease in efficacy was noted over the 7-day period.[a]

There are potential drawbacks to topical serum usage in veterinary medicine. It is possible to use autologous serum, although the volume that can be obtained varies based on patient size. Additionally manual or chemical restraint may be needed to draw blood for serum; this may not be safe for the patient in question. Healthy donor animals may be available although concern for the transmission of pathogens exists, particularly viral agents with feline donors. Additionally, since no preservative is included with serum, the possibility of bacterial contamination exists.[84] Finally a human patient using topical

autologous serum developed a peripheral corneal infiltrate in the superficial corneal stroma that was later determined to be immunoglobulin deposition (IgA, IgG, IgM); serum was suspected to be the source of the immunoglobulins.[86]

2.6 Plasma

Plasma is another component of blood that has received considerable attention in the human literature for use in various ocular conditions. Plasma is the supernatant of blood obtained after anticoagulated blood has been centrifuged, leaving red and white blood cells behind. Plasma contains clotting factors and platelets, which is what differentiates it from serum. Platelets contain many growth factors including EGF, PDGF and TGF-β which are released from activated platelets; plasma also contains fibrin and fibronectin, which are important factors for cell adhesion and migration.[87] There are various iterations of plasma available, including PRP, FFP and platelet releasate (a product where platelets are activated to release their contents after which the platelet remnants are removed). The fraction of plasma with greater numbers of platelets compared to normal plasma, where the increased concentration of platelets is achieved through centrifugation steps, is PRP.[87] Another form of plasma, E-PRP has been described for use both as a topical drop and as a 'clot' for ocular use. E-PRP is plasma located nearest the red blood cells in a centrifuged tube and has a platelet concentration of 1-3 times that of blood.[87,88] The concentration of platelets in blood ranges from 150,000 to 450,000 per microliter; in contrast PRP drops have a platelet count of 800,000/μL and PRP clots have platelet counts of 4,000,000/μL.[89] PRP clots are formed when PRP is mixed with thrombin and calcium, which causes a gel to form.[89] RFRP is plasma with higher fraction of growth factors; after centrifugation, plasma can be divided into 2 or 3 fractions. The first is closest to the red blood cells and has more growth factors and platelets, the second is just above the first and is higher in migratory factors (fibronectin, vitamin A) and the third fraction is uppermost, with all the factors but at

lower concentrations.[90] Platelet releasate is a plasma product where platelets are activated in vitro with thrombin to release their growth factors, centrifugation then removes any platelets, leaving behind a growth factor rich supernatant.[91]

In the human literature, plasma has been applied as an eye drop, as a subconjunctival injection and in clot form to treat a wide variety of corneal conditions, including chemical burns, corneal perforations, dry eye, and non-healing ulcers.[88-90,92-94] The majority of the research in the human field has focused on the role of regenerative factors in plasma, rather than its anti-collagenase effects. Topical PRP promoted faster epithelialization in cases of corneal chemical injury compared to standard treatment with topical antibiotics, topical steroids and other topical medications; subconjunctival RFRP (in addition to conventional treatment with topical medications including topical steroids and antibiotics) reduced the time to corneal and conjunctival epithelialization in patients with ocular alkali burns compared to those treated with conventional treatment alone .[90,92,93] In a study where patients with dry eye were treated with autologous PRP drops, patients reported a significant improvement in symptoms.[94] In another clinical study, autologous PRP drops or clots were used to treat patients with non-healing corneal ulcers, using a decrease in the size/depth of the ulcer as an outcome measure and 38/40 eyes were noted to improve over 3-45 days.[89] E-PRP has been investigated as a way to treat corneal perforations. E-PRP was mixed with calcium chloride to activate the platelets and form a solid clot; the clot was then placed in the perforation and covered with an autologous fibrin membrane in 11 patients. In all 11 cases, the perforation was sealed, with no recurrence of perforation over a 3 month follow-up period.[88]

Several studies have compared serum to plasma, with regards to the epitheliotrophic capacity of each product. Epithelial cell proliferation is enhanced by FGF, HGF and PDGF.[91] The migration and proliferation of epithelial cells is enhanced by EGF.[91] Migration of corneal epithelial cells is also enhanced by fibronectin; vitamins A and E affect cell differentiation and survival, respectively.[91] Hartwig et al. compared serum to platelet releasate in an in vitro corneal epithelial model.[91] They found that serum had higher levels of vitamins A and E, fibronectin and HGF, while platelet releasate had higher levels of EGF, FGF, TGF-β and PDGF.[91] In the same study, they note that serum supported epithelial cell differentiation and migration slightly better while platelet releasate significantly supported cell growth.[91] In 2005, Hartwig et al. compared FFP to serum in an in vitro corneal cell model, this time comparing the levels of PDGF, EGF, TGB-β, and Vitamin A.[95] In these results, serum had higher levels of EGF, TGF- β, PDGF and vitamin A; in contrast to their previous report, serum was superior to FFP at encouraging cell differentiation, growth and migration.[95] In 2006 another study comparing epitheliotrophic factors was published in which serum, FFP and platelet releasate were evaluated.[96] In this study, the levels of TGF-β, EGF, PDGF, vitamins A and E, and fibronectin were measured and compared in a corneal epithelial cell culture model.[96] Their results showed that proliferation of epithelial cells was better with platelet releasate (which had higher levels of EGF, PDGF and TGF- β) but cell differentiation and migration were supported by serum (which had higher levels of vitamins A and E and fibronectin).[96] They concluded that FFP was not likely to be of benefit in treating corneal disease. Again, this research was not focused on the anti-collagenase efficacy of any of the plasma products and no quantification of α-2

macroglobulin was measured.[96] Interestingly a single report has noted that the concentration of α-2 macroglobulin in human API-PRP was increased 5-10 fold compared to the α-2 macroglobulin concentration of blood.[b]

In veterinary medicine, there is significantly less information regarding the use of topical plasma. In general, plasma may hold some benefits over the use of serum as an anti-collagenase, primarily that it is available commercially and this could provide a steady supply for clinical use. Additionally, commercially available plasma is obtained from animals that have been tested for communicable diseases. A recently published abstract detailed a comparison of canine serum, plasma and FFP and their effects in vitro in a commercial gelatinase-collagenase assay.[97] The results of this study showed that all 3 blood derivatives significantly blocked collagenase and gelatinase activity in the in vitro model but that serum was more effective than plasma.[97] Another study compared the anti-gelatinase efficacy of canine serum, FFP, freeze-thaw cycled plasma and Solcoseryl using a commercially available gelatinase activity assay.[98] Solcoseryl is an ultrafiltrate of calf blood which is protein free and has been proposed to improve wound healing.[98] In this study, the plasma and serum showed similar blockage of MMP 2 and 9, while Solcoseryl significantly blocked MMP-9 but not MMP-2.[98] To the authors knowledge, no investigations into the anti-collagenase efficacy of feline or equine plasma have been performed.

2.7 Conclusions

The volume of evidence for use of topical serum as an anti-collagenase is convincing despite the fact that there is not a great deal of information regarding how serum for topical use should be stored. In addition, little research has been performed assessing the effects that storage may have on serum's anti-collagenase efficacy. To the authors knowledge, no previous research has evaluated interspecies use of serum. This research serves to provide more concrete data to guide clinicians on how to store serum for clinical use, as well as providing the first investigation into interspecies use of serum. Finally, this research compares the in vitro anti-collagenase efficacy of serum to plasma in cats, dogs and horses, which has potential implications for providing a stable supply of an effective topical anti-collagenase agent to veterinary patients.

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Footnotes

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CHAPTER 3. THE EFFECTS OF INTERSPECIES USE AND STORAGE CONDITIONS ON THE IN VITRO ANTI-COLLAGENASE EFFICACY OF SERUM

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Supported by a Veterinary Clinical Sciences Graduate Student Competitive Research Grant.

Presented in abstract form at the 45th Annual Meeting of the American College of Veterinary Ophthalmology, Ft. Worth, October 2014

The authors thank Dr. Lynn Guptill and Anisa Dunham for technical assistance.

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3.2 Abstract

Objective−To evaluate interspecies effects, storage time, and temperature on the anti-collagenase efficacy of canine, feline and equine serum in an in vitro corneal degradation model. *Samples*−Normal corneas from recently euthanized dogs, cats and horses; serum from healthy dogs, cats, and horses. *Procedures*− Serum was pooled by species and used fresh or stored for 30, 90, or 180 days at both -20°C and -80°C. Corneas were collected and stored at -80°C. Sections of cornea were dried, weighed and incubated in saline with clostridial collagenase and serum (homologous or heterologous) from each time/temperature point. Corneal damage was assessed by percent corneal weight loss and hydroxyproline (HP) concentration compared to controls. *Results*−Addition of serum resulted in significantly less percent corneal weight loss compared to positive controls ($P<0.001$). Storage time ($P=0.074$) and temperature ($P=0.526$) did not affect percent corneal weight loss, while interspecies cornea/serum combinations ($P=0.028$) did affect percent corneal weight loss. The inclusion of feline and equine sera significantly reduced HP concentration ($P<0.001$) compared to positive controls. Significantly more HP was present in samples incubated with serum stored for 90 days compared to all other time points ($P<0.001$) while temperature did not significantly affect HP concentrations ($P=0.132$). *Conclusions and Clinical Relevance*−Serum was effective at reducing percent corneal weight loss in this model across species. Storage time and temperature did not affect the anti-collagenase efficacy 1to 6 months at -20°C or -80°C without loss of efficacy.

3.3 Introduction

Corneal ulceration is a common clinical problem in domestic species. Corneal ulcers affecting only the epithelium often heal with minimal clinical intervention while ulcers involving the corneal stroma may be more difficult to treat.[1-3] Significant complications can result from stromal collagen destruction which can cause rapid progression of the ulcer to a descemetocele or a corneal perforation.[2-5]

Enzymes capable of degrading protein have been implicated in the progression of human, rabbit, equine and canine corneal ulcers.[1,2, 5-9] The nomenclature for such enzymes varies among sources and the distinctions between names of proteolytic enzymes have historically been confusing.[10, 11] Terms commonly used in the literature include collagenases, MMPs, proteinases, proteases, peptidases, gelatinases and stromeolysins. Often, the terms are used interchangeably but for the purposes of this study, the term collagenase is used to describe an enzyme capable of corneal collagen degradation. Rabbit corneal collagenase, described as a tissue collagenase as it cleaves collagen into ¾ and ¼ fragments, has been implicated in the ulceration of rabbit corneas; human corneal collagenase was shown to breakdown collagen in the same manner.[7] Collagenases can be produced by bacteria, leukocytes or from the growth of corneal epithelium and keratocytes.[1-4, 7-9,12] Regardless of the inciting cause, the progression of stromal ulcers is mediated by collagenases as they breakdown stromal collagen.[1-4,6-8,13,14] In both dogs and horses, levels of MMPs were higher in ulcerated eyes than in the contralateral unaffected eyes and the levels decreased significantly as the ulcerations

healed.[5, 9] As such, inhibition of collagenase can be key to halting the progression of corneal ulceration, minimizing sequelae and promoting corneal healing.[1-3, 6, 8,12, 13]

Many topical ophthalmic agents have been utilized clinically as inhibitors of corneal collagenase, including calcium-EDTA, NAC and tetracycline.[1-4, 6, 14] The use of topical human serum as an effective inhibitor of corneal collagenase has been described.[6, 7, 13] The inhibitory effect of serum was found to be due to α-2 macroglobulin which forms tight complexes with collagenases and inhibits their activity.[6,7,15,16]

In veterinary medicine, autogenous or homologous serum has been used topically for its anti-collagenase properties to treat corneal ulcers involving the stroma.[1-4, 8] Despite the widespread usage of topical serum as an anti-collagenase agent, to the authors' knowledge, there is no information regarding the anti-collagenase efficacy of serum after storage longer than 7 days in human or veterinary medicine, and no comparison of the differences between the use of heterologous and homologous serum.

We hypothesized that serum stored up to 6 months at -20°C or -80°C would have the same anti-collagenase efficacy as fresh serum when evaluated in an in vitro corneal degradation model. Additionally we hypothesized that the anti-collagenase efficacy of heterologous serum would not differ from that of homologous serum.

3.4 Materials and Methods

Corneal Samples−Corneas were collected from recently euthanized dogs, cats and horses from Purdue University Veterinary Teaching Hospital or a local animal shelter. Animals were euthanized for reasons unrelated to this study and had no evidence of corneal disease as determined by examination with a direct light source. The corneas were removed and sectioned into 4 pieces (feline and canine) or 6 pieces (equine) of roughly equal size within 2 hours of euthanasia. Each piece of cornea was stored individually, labeled by species and kept at -80°C until use. Due to the extended nature of the study, corneal sections were stored for varying lengths of time, up to 6 months.

Serum Samples−Serum was obtained from healthy canine (n=4), feline (n=6) and equine (n=4) donors. Cats were anesthetized for blood collection, whereas dogs and horses were manually restrained. Blood was collected into serum separator tubes, allowed to clot and centrifuged to separate the serum. The serum was then removed and pooled by species. The pooled serum was then divided into aliquots and used fresh, or stored for 30, 90 or 180 days, at both -20°C and -80°C. All procedures were approved by the Purdue University Animal Care and Use Committee. Sterile technique was used throughout the experiment but at no time were any cultures performed on serum samples.

In Vitro Corneal Degradation Model−A previous report by Haffner et al.[12] provided the basis for the current experimental design, utilizing a clostridial collagenase to incite corneal degradation. Individual corneal samples were placed in plastic weigh boats and dried in a 40° C oven for 3 hours. After drying, the pre-treatment corneal weight was

recorded. Incubation fluid was prepared by adding 800 U/mL of collagenase derived from *Clostridium histolyticum*[a] to 5 mM calcium chloride in 0.9% sodium chloride. Five mL of the resultant solution was added to a 10 mL tube. Then 500 µL of serum (100 µL/mL) from the appropriate species and storage time/temperature combination was added to each tube. Negative controls consisted of 5 mL of 5 mM calcium chloride in 0.9% sodium chloride only. Positive controls consisted of 5 mM calcium chloride in 0.9% sodium chloride with 800 U/mL of clostridial collagenase but no serum. Negative and positive controls were included for each experimental condition. The dried corneal samples were added to the tubes and incubated with agitation at 40°C for 4 hours. After incubation, 1.8 mL of incubation fluid was collected from each tube and stored at -80°C for HP analysis at a later date. The cornea was then collected by pouring the remaining solution through Whatman filter paper[b]. The corneas were then dried at 40°C for 3 hours, weighed and the post treatment percent corneal weight loss recorded.

Corneal samples from each species were incubated in homologous serum and two heterologous sera. Each experiment was carried out in triplicate. Experimental conditions are summarized below.

Homologous serum−Corneas from each species were incubated with pooled homologous serum stored at -20°C and -80°C for time periods of 0 (fresh serum), 30, 90 or 180 days.

Heterologous serum−Corneas from each species were incubated in heterologous serum stored at -20°C and -80°C for time periods of 0 (fresh serum), 30, 90 or 180 days.

HP Assay− As HP is a known breakdown product of collagen and has been used previously in a similar study, HP concentrations were used as a second means of analysis of corneal degradation.[12] A commercial HP spectrophotometric assay kit[c] was used to measure HP concentration in µg/mL in the incubation media for all samples and controls. The analyses were performed according to kit instructions with two modifications. Modifications included that samples were diluted 1:2 with ultrapure water prior to analysis to ensure HP concentrations measured would fit on the standard curve and samples were centrifuged for 10 minutes at 10,000 rpm. A new standard curve calculated for each plate.

Statistical Analysis−A Shapiro-Wilk statistic was used to test for normality on percent corneal weight loss and HP. Mean and standard deviation (SD) were reported if the distribution was approximately normal, otherwise median and range were reported. A general linear model was used to compare the means across groups if the distribution was approximately normal, otherwise the Kruskal-Wallis test was used. Pairwise comparisons with Bonferroni adjustment were performed if the overall F test of the general linear model or Kruskal-Wallis test was significant. A *P* value of <0.05 was considered significant. Statistical software was used for all analyses.[d]

3.5 Results

In Vitro Corneal Degradation Model−This model was effective at producing corneal weight loss by collagen degradation. The percent corneal weight loss for feline, canine and equine corneas incubated in feline, canine and equine sera, including positive and negative controls are shown in Table 3.1.

Serum was effective at reducing percent corneal weight loss compared to positive controls in this study. Across species, inclusion of feline, canine and equine serum lessened corneal weight loss by 38 percent ($P<0.001$), 11 percent ($P<0.001$) and 25 percent ($P<0.001$) respectively compared to mean positive control samples. Overall, corneas incubated in feline serum had the least percent corneal weight loss compared to canine or equine serum ($P<0.001$). Furthermore, the percent corneal weight loss differed significantly among different cornea/serum species combinations ($P=0.028$) Significant differences are summarized in Table 3.1.

No significant difference in percent corneal weight loss occurred for corneas incubated with serum stored for 0, 30, 90 or 180 days ($P=0.074$) (Table 3.2). No significant difference in percent corneal weight loss occurred for corneas incubated in fresh serum or serum stored at -20°C or -80°C ($P=0.526$) (Table 3.3).

HP Assay−Higher concentrations of HP were measured in incubation fluid from positive control samples compared to fluid from negative control samples. Across species, inclusion of feline and equine sera significantly reduced HP concentration (both

P<0.001) compared to positive control samples. Inclusion of canine serum did not reduce HP concentration compared to positive controls (*P*=0.229). HP concentrations for feline, canine and equine corneas incubated in feline, canine and equine sera, including positive and negative controls are summarized in Table 3.4. The amount of HP was significantly lower for canine corneas incubated in feline serum compared to canine corneas incubated in canine serum (*P*=0.007) but no other cornea/serum combinations were significantly different (Table 3.4).

The HP concentration of incubation fluid from corneas incubated with serum stored for 90 days was significantly higher than that of fresh serum (*P*<0.001), 30 day stored serum (*P*<0.001) and 180 day stored serum (*P*<0.001) (Table 3.5). No significant difference in the amount of HP was detected for corneas incubated with fresh serum or serum stored at -20°C or at -80°C (p=0.132, Table 3.6).

Corneal weight loss was weakly correlated to HP concentration (Spearman's rho=0.12; *P*=0.096), where an increase in percent corneal weight loss corresponded to an increase in HP concentration (Figure 1).

3.6 Discussion

Sera from feline, canine, and equine species were all effective at reducing corneal weight loss in this in vitro model. Feline and equine sera were effective at reducing HP concentration. Neither storage time nor temperature resulted in a significant difference in percent corneal weight loss or HP production. Interspecies use of serum did have a significant effect on percent corneal weight loss, with feline serum being the most protective regardless of the species of the corneal sample.

Proteinases are a group of enzymes that break down proteins; they can be subdivided further into cysteine, aspartic, serine or metalloproteinases.[10, 17] The MMPs are a group of zinc dependent enzymes (which include collagenases) capable of degrading collagen and other matrix proteins.[1, 2,18, 19] In the cornea, MMPs are required for general repair and remodeling of the stroma; they are balanced by endogenous enzyme inhibitors. Dysregulation of this system can lead to uncontrolled collagen breakdown.[2, 19,20] This breakdown of corneal collagen, or keratomalacia, results from an increase in collagenases from various sources, both endogenous (corneal epithelial cells, leukocytes, tear film) and exogenous (bacterial or fungal organisms).[1-4,7,8, 12,20]

Many compounds have been investigated for their utility as corneal anti-collagenolytic agents. Collagenase inhibitors can be divided into those that are specific or nonspecific. Specific inhibitors block only one type of proteinase (serine for example) while nonspecific inhibitors such as α macroglobulins can block all 4 types of proteinases.[16, 17 , 21] Topical agents, which have been effective collagenase inhibitors,

include NAC, EDTA, tetracycline, tetanus antitoxin and autogenous serum.[1, 2, 4,8,12] Both EDTA and NAC exert their anti-collagenolytic effect by chelating zinc, and/or calcium, which are required cofactors for the MMPs.[2-4,22] Tetracycline, used topically or systemically, chelates zinc and calcium, inhibits the breakdown of endogenous anti-trypsin and may inhibit leukocyte migration.[2-4,22] Serum contains α-2 macroglobulin, a non-specific collagenase inhibitor, and alpha-1 antitrypsin.[1-4,6-7,13-14,17] Human alpha-1 antitrypsin was found not to inhibit human corneal collagenase, although it did inhibit rabbit corneal collagenase, whereas α-2 macroglobulin (in human and rabbit sera) was inhibitory.[6, 7,13] Human α-2 macroglobulin, a protein produced in the liver, accounts for 8-10 percent of serum proteins and inhibits both endogenous and exogenous collagenases by forming tight complexes with the collagenase; kinetic studies have demonstrated that α-2 macroglobulin is an important inhibitor of collagenases.[2,13,16, 17,21] Wooley et al.[23] found that greater than 90% of the anti-collagenase effect of serum was due to α-2 macroglobulin. Haffner et al.[12] found no significant difference in the anti-collagenase effects of equine serum, tetanus antitoxin and NAC in an in vitro corneal degradation model and Ollivier et al.[8] found that doxycycline, EDTA, NAC, ilomostat and equine serum all inhibited latent and active forms of MMP. Serum has several potential advantages over other anti-collagenases: it is well tolerated when used topically on the eye and can be readily obtained from healthy donors. As such, serum is often used in both human and veterinary practice as an anti-collagenase.

Despite its widespread use, few guidelines exist regarding the storage of serum for topical use and of those that have been published, few provide data to support the

recommendations regarding storage time or temperature.[24-26] One report notes possible storage protocols: store fresh serum at 4°C for no more than 16 hours or freeze serum at -20°C and store it for up to 3 months; serum can be stored frozen for 6 months but once in use should be disposed of after a day.[26] Liu et al.[24] investigated the effects that preparation methods have on epitheliotrophic factors present in human serum. Based on their findings, they suggested that serum be allowed to clot at room temperature for 2 hours and subsequently be centrifuged for 15 minutes at 3,000 g. Once obtained the serum should be diluted 1:4 with balanced saline solution and stored at -20°C for no longer than 3 months, although they did not investigate effects of storage time or temperature. Tsubota et al.[25] investigated the effects of storage on several components of serum for ocular use, comparing concentrations of epithelial growth factor, vitamin A and TGF-β1 in fresh serum to that which had been stored for 1 month at 4°C or 1 and 3 months at -20°C. No differences in concentrations of the studied components were found among the storage times, but there was no investigation of effect of storage on α-2 macroglobulin concentrations.

Published storage recommendations for veterinary ophthalmic use have varied. Serum can reportedly be stored indefinitely if frozen, or stored at room temperature or refrigerated and replaced every 8 days due to concerns regarding microbial contamination.[2,4] A previous study by Brooks et al.[e] reported that equine serum stored at -18°C, 4°C and at 23°C for 7 days had no decrease in its ability to inactivate MMPs in equine tears over a 7 day period, nor was there a difference in inhibitory activity between the different temperatures. The results of the current study demonstrated that neither the

storage time (up to 180 days) nor the storage temperatures evaluated (-20°C or -80°C) had a significant effect on the percent corneal weight loss in an in vitro corneal ulcer model. This is consistent with the results of Brooks et al.[e] and Tsubota et al.[25] but our study evaluated a longer storage time period and investigated only anti-collagenase efficacy. Since our study did not evaluate serum stored at room temperature or 4°C we cannot compare our results with regards to these temperature conditions. Further studies are needed to evaluate the maximum time that serum could be stored at various temperatures without loss of efficacy.

Quantification of HP, a breakdown product of collagen, has proven useful for investigating collagen degradation.[27] In the study by Haffner et al.[12] corneal weight loss and HP were highly correlated (r =0.80), where an increase in corneal weight loss corresponded to increased HP concentration. This is in contrast to our results, where percent corneal weight loss and the amount of HP were only weakly correlated (Spearman's *rho* =0.12), although an increase in percent corneal weight loss did correspond to increased HP. Inclusion of feline and equine serum overall reduced the amount of HP in incubation media, while inclusion of canine serum did not, indicating that more collagen breakdown occurred in the presence of canine serum compared to equine and feline sera. Storage temperature did not significantly affect the HP concentration. The HP concentration from samples incubated in serum stored for 90 days was significantly higher compared to serum stored for 0, 30 and 180 days. Clinically, this result may not be relevant, as no reason for this discrepancy could be found.

Additionally, corneas incubated in serum stored for 90 days did not have significantly more weight loss compared to other time points.

As expected, the inclusion of serum in this in vitro model resulted in decreased percent corneal weight loss compared to positive controls. Interestingly, feline serum was the most protective across all the species of cornea on which it was tested. Knowing that α-2 macroglobulin is the molecule responsible for the anti-collagenase effect and that it comprises 8-10 percent of human serum proteins, it is tempting to speculate that feline α-2 macroglobulin may be present in greater concentrations or has superior binding capacity compared to canine or equine α-2 macroglobulin, but this has not been investigated. Future research into characterizing α-2 macroglobulin of different species and whether there are species differences in its binding capacity should be undertaken. Predicted protein sequences for Isoforms X1 and X2 of canine α-2 macroglobulin, feline α-2 macroglobulin and equine α-2 macroglobulin were identified on the National Center for Biotechnology Information website.[28] The protein sequence was also available for human α-2 macroglobulin. A Basic Local Alignment Search Tool (BLAST) search and comparison were performed: The predicted α-2 macroglobulin isoforms x1 and x2 (canine) have 99% conserved domains, Isoform x1 and x2 have 82% conserved domains compared to the horse and 86% conserved domains compared to the cat.[29] The feline predicted α-2 macroglobulin has 86% conserved domains compared to the dog x1 and x2 and 81% conserved domains compared to the horse.[29] The predicted α-2 macroglobulin of the horse has 82% conserved domains to canine isoforms x1 and x2, and 81% conserved domains to the cat.[29] The human α-2 macroglobulin has 81% conserved to the

horse, 80% compared to canine isoforms X1 and X2, and 80% compared to the feline.[29] These similarities suggest that the α-2 macroglobulins of the species studied should perform similarly, although this cannot be definitively stated. A recommendation of preferentially using feline serum topically cannot be made without further study. Additionally, as feline patients may be infected with contagious viruses, the use of feline serum from untested cats on other feline patients cannot be recommended.

It is also possible that differences in the corneas themselves, rather than the inhibitory effects of serum, are responsible for the interspecies differences in percent corneal weight loss. Previous investigations into the corneas of domestic species have shown some differences in corneal collagen type and thickness with respect to age, sex and species.[30-33] As this study evaluated percent corneal weight loss, absolute corneal weight and composition were not important parameters, although we cannot say with certainty that collagen type could not play a role.

This study demonstrated serum can be stored for up to 180 days at either -20° C or -80° C with no loss of anti-collagenase efficacy in this in vitro model. Feline serum may be most efficacious but further research into differences in feline, canine and equine α-2 macroglobulins are needed.

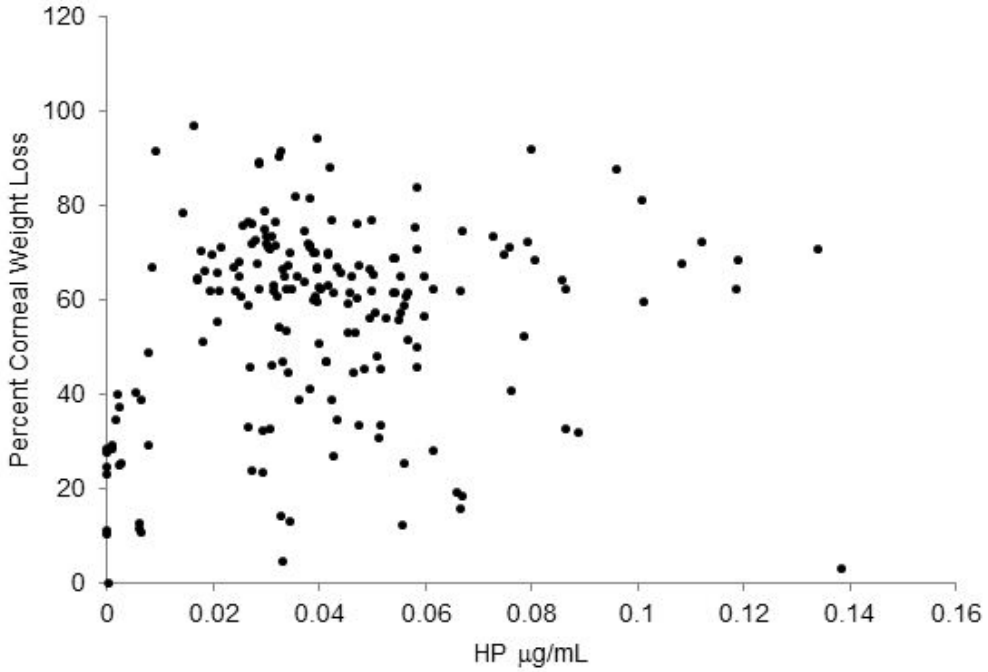

Figure 3.1 Scatter plot of hydroxyproline (HP) concentration versus percent corneal weight loss. HP concentration was weakly correlated to percent corneal weight loss (Spearman's rho=0.12; P=0.096

Table 3.1 Mean percent corneal weight loss for feline, canine or equine corneas incubated with feline, canine or equine serum. Overall, corneas incubated in feline serum had the least percent corneal weight loss compared to canine or equine serum (*, P<0.001).). Feline corneas incubated in feline serum had significantly less percent corneal weight loss than feline corneas incubated in canine serum (†, *P*=0.002). Canine corneas incubated in feline serum had significantly less percent corneal weight loss than canine corneas incubated in canine serum (ǂ, *P*=0.008), Equine corneas incubated in feline serum had significantly less percent corneal weight loss than equine corneas incubated in canine serum (§, *P*<0.001) and equine corneas incubated in equine serum (||, *P*=0.019). Equine corneas incubated in equine serum also had significantly less percent corneal weight loss than equine corneas incubated in canine serum (¶, *P*=0.004).

Mean (SD) percent corneal weight loss

		Cornea		
		Feline	Canine	Equine
	Negative Control	6 (4)	6 (5)	4 (2)
	Positive Control	77 (10)	93 (5)	72 (12)
Serum	Feline	52 (16)*,†	55 (17)^{*,ǂ}	24 (15)$^{*,§,\|\|}$
	Canine	74 (10)†	74 (17)^{ǂ}	62 (12)§
	Equine	66 (10)¶	63 (6)	39 (16)$^{\|\|,¶}$

Table 3.2 Mean (SD) percent corneal weight loss relative to serum storage time, across all species cornea/sera combinations. Storage time did not significantly affect percent corneal weight loss. (P=0.074)

Day	Percent Weight Loss (SD)
0	63 (13)
30	48 (22)
90	58 (19)
180	60 (22)

Table 3.3 Mean (SD) percent corneal weight loss relative to storage temperature, across all species cornea/sera combinations. Storage temperature did not significantly affect percent corneal weight loss. (P=0.526)

Temperature	Percent Weight Loss (SD)
Fresh	63 (13)
-20	56 (23)
-80	55 (20)

Table 3.4 Median HP concentration (µg/mL) of the incubation media using heterologous sera. Across species, inclusion of feline* and equine† sera significantly reduced HP concentrations (P <0.001) compared to positive control samples. Inclusion of canine serum did not reduce HP concentration compared to positive controls (P=0.229). Canine corneas incubated in feline serum had significantly less HP than canine corneas incubated in canine serum (ǂ, P<0.05)

		Cornea		
		Feline	Canine	Equine
	Negative Control	0.0003 (0-0.003)	0 (0-0.009)	0.0014 (0-0.006)
	Positive Control	0.04 (0.03-0.09)*,†	0.04 (0.02-0.11)*,†	0.08 (0.04-0.149)*,†
Serum	Feline	0.040 (0-0.086)*	0.025 (0-0.056)* ǂ	0.036 (0-0.138)*
	Canine	0.032 (0.009-0.112)	0.042 (0.028-0.101) ǂ	0.055 (0.027-0.134)
	Equine	0.039 (0.016-0.058)†	0.033 (0.008-0.060)†	0.038 (0.005-0.087)†

Table 3.5 Median (range) HP concentration with respect to storage time. Significantly more HP was present in fluid from corneas incubated in serum stored for 90 days* compared to fresh (P<0.001), 30 (P<0.001) or 180 (P<0.001) day serum.

Storage Time (days)	HP (µg/mL)
0	0.034 (0.005-0.086)
30	0.033 (0-0.079)
90	0.055 (0.022-0.138)*
180	0.032 (0.016-0.086)

Table 3.6 Median (range) HP concentration with respect to storage temperature. There was no significant difference in HP concentrations with respect to storage temperature. (P=0.132)

Storage Temperature	HP (µg/m)
Fresh	0.034 (0.005-0.086)
-20º C	0.040 (0-0.134)
-80º C	0.039 (0-0.138)

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Footnotes

a. Collagenase from *Clostridium histolyticum,* type XI, Sigma Aldrich, St Louis , Mo.

b. Whatman filter paper, Sigma Aldrich, St Louis, Mo.

c. Hydroxyproline assay kit, Sigma Aldrich, St Louis, Mo.

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CHAPTER 4. COMPARISON OF THE IN VITRO ANTI-COLLAGENASE EFFICACY OF SERUM AND PLASMA IN A CORNEAL DEGRADATION MODEL

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The authors thank Dr. Lynn Guptill and Anisa Dunham for technical assistance.

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4.2 Abstract

Objective−To compare the anti-collagenase efficacy of fresh feline, canine and equine serum to plasma in an in vitro corneal degradation model. *Samples*−Normal corneas from recently euthanized dogs, cats and horses; serum and plasma from healthy dogs, cats, and horses. *Procedures*− Serum and plasma were pooled by species and used fresh in the in-vitro model. Corneas were collected and stored at -80°C. Sections of cornea were dried, weighed and incubated in saline with clostridial collagenase and homologous fresh serum or plasma. Corneal degradation was assessed by percent corneal weight loss and hydroxyproline (HP) concentration compared to positive and negative controls. *Results*− Serum and plasma significantly reduced percent corneal weight loss compared to positive controls in this model. No significant difference was found between feline (P=0.579), canine (P=0.249) or equine (P=0.406) corneas incubated with serum or plasma with regards to percent corneal weight loss. Canine serum and plasma significantly reduced HP levels while inclusion of feline and equine serum or plasma did not, compared to positive controls. HP levels were moderately correlated with percent corneal weight loss for feline sample (P=0.002), weakly correlated for equine samples (P=0.096) but were not correlated with percent corneal weight loss for canine samples (P=0.842). *Conclusions and Clinical Relevance*− In this model, the anti-collagenase efficacy of fresh feline, canine and equine serum was not different to that of plasma. Plasma should be an acceptable substitute for topical serum in the clinical treatment of keratomalacia.

4.3 Introduction

The development of corneal ulceration is a frequent problem in domestic species. The progression of corneal ulcers via degradation of corneal stroma is known to be mediated by collagenases, enzymes that can come from infectious organisms, leukocytes or corneal epithelial cells.[1-3] Inhibition of these destructive enzymes can thus be crucial to preventing further progression of corneal degradation.[2]

Topical serum has been utilized in human and veterinary medicine for various ocular conditions. In human medicine, topical serum is utilized to treat dry eye, persistent epithelial defects, neurotrophic keratopathy, superior limbic keratoconjunctivitis and Mooren's ulcers.[4,5] Umbilical cord serum has also been investigated as a treatment for acute ocular chemical burns.[6] In veterinary medicine, topical serum has been widely used as an anti-collagenase when treating corneal stromal ulcers in an attempt to slow or arrest their progression.[2,3,7] The anti-collagenase effect of topical serum is attributed to the presence of α-2 macroglobulin, a serum protein which complexes with and inhibits corneal collagenase.[8,9]

Plasma has also been investigated in human medicine for potential benefits when used topically. Plasma has some possible benefits over serum, namely that plasma is commercially available and therefore perhaps easier to obtain; plasma also contains clotting proteins and platelets, which provide sustained release of growth factors.[10,11] Hartwig et al.[12] compared serum and fresh frozen plasma (FFP) in a human corneal epithelial cell model to determine their relative effects on various aspects of corneal

wound healing and found that serum was superior to FFP with regards to enhancing epithelial cell growth, differentiation and migration. Numerous other studies have evaluated FFP, platelet rich plasma (PRP) and platelet releasate (a product where the growth factors in platelets are released by thrombin stimulation and the remaining platelet membranes are removed by centrifugation) for treatment of ocular surface defects, ocular burns, non-healing corneal ulcers, corneal perforations and dry eye and found clinical improvement with their use.[10-11,13-19]

Despite previous research into the effects of plasma and its constituents on corneal conditions in humans, there has been little investigation into the anti-collagenase efficacy of plasma in veterinary medicine. Two previous studies[20, 21] have evaluated canine plasma in vitro in comparison to serum with regards to their anti-collagenase efficacy but to the authors' knowledge, no studies have evaluated the anti-collagenase efficacy of feline or equine plasma compared to serum. The purpose of this study was to compare the anti-collagenase efficacy of fresh feline, canine and equine serum to that of plasma in an in vitro corneal degradation model.

4.4 Materials and Methods

Corneal Samples−Corneas were collected from recently euthanized dogs, cats and horses from the Purdue University Veterinary Teaching Hospital or a local animal shelter. Animals were euthanized for reasons unrelated to this study and had no evidence of corneal disease as determined by examination with a direct light source. The corneas were removed and sectioned into 4 pieces (feline and canine) or 6 pieces (equine) of

roughly equal size. Each piece of cornea was stored individually, labeled by species and kept at -80°C until use. Corneal sections were stored for time periods of 1-18 months before use. Storage times for corneal samples were evenly distributed among control and test groups.

Serum and Plasma Samples−Whole blood was obtained from healthy canine (n=4), feline (n=4) and equine (n=4) donors. The dogs and cats were employee owned animals, whereas the horses were Purdue University owned teaching animals. Cats were anesthetized for blood collection, whereas dogs and horses were manually restrained. To obtain serum, blood was collected into serum separator tubes[a], allowed to clot, and centrifuged to separate the serum. The serum was then removed and pooled by species. To obtain plasma, the blood was collected into lithium heparin tubes[b] and centrifuged to separate the plasma. To avoid confounding the results, EDTA was not used as an anticoagulant as EDTA has anti-collagenase properties. The plasma was then removed and pooled by species. The pooled serum and plasma were then used within 24 hours. All procedures were approved by the Purdue University Animal Care and Use Committee. Employees signed written consent forms authorizing the use of their pets for blood collection. Sterile technique was used throughout the experiment but no bacterial cultures were performed on the pooled serum or plasma.

In Vitro Corneal Degradation Model− Individual corneal samples were placed in plastic weigh boats and dried in a 40° C oven for 3 hours. After drying, the pre-treatment corneal weight was recorded. Incubation fluid was prepared by adding 800 U/mL of

collagenase derived from *Clostridium histolyticum*[c] to 5 mM calcium chloride in 0.9% sodium chloride. Five mL of the incubation fluid was added to a 10 mL tube containing the corneal sample. Then 500 µL of serum or 500 µL of plasma from the appropriate species was added to the incubation fluid so that sections of cornea were incubated with either homologous serum or homologous plasma. Negative controls consisted of 5 mL of 5 mM calcium chloride in 0.9% sodium chloride only. Positive controls consisted of 5 mM calcium chloride in 0.9% sodium chloride with 800 U/mL of clostridial collagenase but with neither serum nor plasma. For each species, 15 corneal samples were incubated individually with serum and 15 corneal samples were incubated individually with plasma. Positive and negative control samples for each species were run in triplicate. Tubes were incubated with agitation at 40°C for 4 hours. After incubation, 1.8 mL of incubation fluid was collected from each tube and stored at -80°C for HP analysis at a later date. The cornea was then collected by pouring the remaining solution through Whatman filter paper.[d] The post treatment corneas were then dried at 40°C for 3 hours, weighed and the post treatment percent corneal weight loss recorded.

HP Assay−As a second method of evaluating corneal collagen degradation, HP concentration in the incubation media was evaluated using a commercial HP spectrophotometric assay kit[e]. The analyses were performed according to kit instructions with two modifications: samples were diluted 1:2 with ultrapure water prior to analysis to ensure HP concentrations measured would fit on the standard curve and samples were centrifuged for 10 minutes at 10,000 rpm. A new standard curve was calculated for each plate per kit instructions.

Statistical Analysis- Kruskal-Wallis tests were used to compare percent corneal weight loss and HP levels among serum, plasma and positive controls. Dunn's tests were performed for pairwise comparisons following a significant Kruskal-Wallis test (i.e., $P<0.05$). Correlation between percent corneal weight loss and HP concentration was assessed by Spearman correlation coefficient. Only serum and plasma samples were analyzed for correlation, positive and negative control sample results were not included in this calculation.

4.5 Results

In Vitro Corneal Degradation Model−This model was effective at producing corneal weight loss by collagen degradation. The mean (SD) percent corneal weight loss for feline, canine and equine positive controls were 85% (3), 100% (4) and 76% (13) respectively while the mean (SD) percent corneal weight loss for feline, canine and equine negative controls were 4% (2), 4% (4) and 1% (2) respectively. The mean (SD) percent corneal weight loss for feline, canine and equine corneas incubated in serum were 62% (7), 74% (15) and 38% (12) respectively. The mean (SD) percent corneal weight loss for feline, canine and equine corneas incubated in plasma were 60% (10), 60% (27) and 41% (14), respectively.

Serum and plasma were both effective at reducing percent corneal weight loss compared to positive controls in this study. Inclusion of feline, canine or equine serum, on average, reduced corneal weight loss by 23% ($P=0.012$), 26% ($P=0.018$) and 38% ($P=0.005$) compared to respective positive control samples. Inclusion of feline, canine or

equine plasma, on average, reduced corneal weight loss by 25% (*P*=0.004), 40% (*P*=0.002) and 35% (*P*=0.019) respectively compared to respective positive control samples. There was no significant difference in corneal weight loss between corneas incubated with serum or plasma: feline (*P*=0.579), canine (*P*=0.249) or equine (*P*=0.406) (Figure 4.1).

HP Assay– Higher concentrations of HP were measured in incubation fluid from positive control samples compared to fluid from negative control samples. The mean (SD) HP level (μg/mL) for feline, canine and equine positive controls were 0.04 (0.004), 0.04 (0.04) and 0.04 (0.02), respectively while the mean (SD) HP level (μg/mL) for feline, canine and equine negative controls were 0.001 (0.001), 0.004 (0.007), and 0.007 (0.01), respectively. The mean (SD) HP level (μg/mL) for feline, canine and equine corneas incubated in serum were 0.04 (0.01), 0.01 (0.01) and 0.06 (0.03), respectively. The mean (SD) HP level (μg/mL) for feline, canine and equine corneas incubated in plasma were 0.04 (0.01), 0.01 (0.01) and 0.06 (0.03), respectively.

The inclusion of canine serum (*P*=0.028) or canine plasma (*P*=0.009) significantly reduced HP concentration compared to positive controls (Figure 4.2). There was no significant difference in HP concentration between corneas incubated with canine serum or canine plasma (*P*=0.497). There was no significant difference in HP concentrations between positive control samples and samples incubated with feline serum or plasma (*P=0.480*) or equine serum or plasma (*P=0.846*). (Figure 4.2).

Correlation- Spearman's correlation coefficients were 0.54 (*P*=0.002), 0.04 (*P*=0.842) and 0.31(*P*=0.096) for feline, canine and equine samples, respectively (Figure 4.3 A-C).

4.6 Discussion

In this study plasma and serum were equally effective in all species tested at reducing corneal weight loss in vitro. This finding suggests that either plasma or serum could be used topically on an eye with keratomalacia with equal benefit. Serum has been used extensively to treat many different ocular diseases in both humans and domestic species.[2-5,7] The beneficial effects of topical serum can be attributed to constituent growth factors, vitamins, neuropeptides and anti-collagenases.[4] A number of these, including EGF, TGF-β, fibronectin and substance P, promote migration and adhesion of corneal epithelial cells as well as corneal stromal and epithelial repair.[4] Vitamin A can help to stop corneal squamous metaplasia, while α-2 macroglobulin exhibits anti-collagenase effects. Fibronectin is a supportive factor in cellular migration.[4] Serum also contains immunoglobulins, which can be bacteriostatic, or bactericidal.[4] Many of these factors (EGF, TGF-β1, vitamin A, fibronectin) are present in higher concentrations in serum than in tears.[4] Potential risks of topical serum usage include blood borne disease transmission, the deposition of immunoglobulins in the cornea and contamination of the serum with bacteria.[4-5] Most of the benefits seen in human cases where topical serum has been used have been related to improvement in clinical signs, healing of persistent ulcers and reduced recurrence of ulcers rather than anti-collagenase activity.[4-5] In veterinary

medicine, serum has primarily been used for its anti-collagenase effects rather than for epithelial healing.[2-3,7]

Given its similar composition to serum, plasma has also been studied for ophthalmic usage, although it too is mostly evaluated for its ability to affect corneal epithelial growth and not for its anti-collagenase effect. When activated, platelets in plasma release growth factors and other cytokines, which can contribute to the corneal healing process. Serum has been compared to FFP, and to platelet releasate with regards to their respective corneal epitheliotrophic capacities.[12,17] Hartwig et al.[12] compared the concentrations of EGF, PDGF, TGF-β, fibronectin and vitamin A in serum and FFP; they also assessed the effects of either blood product on corneal epithelial cells in vitro. In that study, serum had significantly higher concentrations of all the factors except for fibronectin, which was present in similar amounts to that in FFP; serum was significantly better at stimulating cell growth, migration and differentiation compared to FFP. In 2006, Liu et al.[17] investigated the concentrations of EGF, PDGF, TGF-β, fibronectin, vitamin A and vitamin E in serum, FFP and platelet releasate. In the Liu study serum had significantly higher levels of fibronectin and vitamins A and E, whereas platelet releasate had significantly higher levels of EGF, PDGF and TGF-β than serum, which had more of the same factors than FFP.[17] In the same study, in vitro corneal cell proliferation was best supported by platelet releasate, followed by serum and then FFP but cell differentiation and migration were best supported by serum.[17] These results are similar to that of an earlier in vitro study, which compared serum with platelet releasate and found that platelet releasate resulted in significantly better cell growth while cell differentiation and

migration were slightly improved with serum.[16] These findings are consistent with what we know about the various growth factors regarding corneal epithelium, for example that PDGF stimulations cell proliferation while fibronectin stimulates cell migration.[16]

Platelet rich plasma specifically has been utilized topically to treat acute corneal chemical burns, dry eye, non-healing corneal ulcers and used in conjunction with autologous fibrin membranes to treat corneal perforations.[10-11,13,15] The PRP can be formulated for eyes in 2 ways - as a topical drop or as a clot, both of which have higher concentrations of growth factors than serum.[11] In one report, the PRP drop had a platelet concentration of 800,000/µL whereas the clot had 4,000,000/µL.[11] The normal circulating concentration of platelets in humans is 150,000 to 450,000/µL.[11] Alio et al.[13] found that topical PRP drops significantly improved the symptoms and clinical signs of human patients with dry eye. In another study, Alio et al.[11] also found that PRP, when used as a topical drop or with surgical placement of a PRP clot under an amniotic membrane (for eyes with impending or current perforations), improved healing of corneal ulcers that had not responded to previous conventional treatment. A recent report details the use of a combination of an autologous PRP clot and an autologous fibrin membrane to surgically treat 11 patients with corneal perforations, with no reported complications and no evidence of relapse or perforation after 3 months, with 7 of 11 eyes eventually receiving corneal transplants.[15]

Plasma and its various iterations are supportive of corneal epithelial healing, but there is little investigation into the anti-collagenase efficacy of plasma. Alio et al.[11] used

reduction of depth of the ulcer as a primary outcome measure but did not specifically investigate anti-collagenase activity. One report noted that the concentration of α-2 macroglobulin in human API-PRP was increased 5-10 fold compared to the α-2 macroglobulin concentration of blood.[f] This suggests that the anti-collagenase activity of PRP could be superior to that of serum, although studies to quantify the amount of α-2 macroglobulin in serum of non-human species would be needed before direct comparisons could be made. One veterinary study evaluated the anti-collagenase activity of fresh canine serum and plasma, as well as frozen canine plasma, with a commercial gelatinase and collagenase kit.[20] In that study serum was superior to either plasma product with regards to decreasing protease activity although all three substances were effective.[20] Another veterinary study compared canine serum, canine FFP, canine freeze-thaw cycled plasma and Solcoseryl (an commercial ultrafiltrate of calf blood which is free of protein) using a gelatinase kit; results showed that Solcoseryl only inhibited matrix metalloproteinase-9, while serum, FFP and canine freeze thawed plasma had comparable levels of inhibition for both matrix metalloproteinase-2 and matrix metalloproteinase-9.[21] These results are similar to the results obtained in the present study, where serum and plasma did not differ in their anti-collagenase efficacy.

With regards to HP levels in the incubation media, higher levels of HP were detected in positive control samples compared to negative control samples. Inclusion of canine serum or plasma resulted in significantly lower levels of HP while the inclusion of feline and equine serum and plasma did not significantly reduce HP levels. Overall, when

comparing the HP levels of incubation fluid for feline, canine and equine corneas, there was no significant difference in the HP levels with serum compared to plasma.

In the current study, HP levels were moderately correlated with percent corneal weight loss for feline samples, moderately correlated with equine samples, but were not correlated with percent corneal weight loss for canine samples. One previous study[1] showed a strong positive correlation between equine corneal weight loss due to collagen degradation and HP levels of the incubation fluid, while a previous study in our laboratory found only weak correlation between percent corneal weight loss and HP levels.[22] The current results, along with our previous results, show that HP levels may vary widely, making their interpretation more difficult.

The results of the current study demonstrate that the anti-collagenase efficacy of plasma and serum do not differ in this in vitro model. This finding suggests that either of the two compounds could be used for treating keratomalacia. Additional studies are needed to determine if the anti-collagenase efficacy of plasma is comparable to serum in vivo and also to determine if PRP or plasma releasate could be of value with regards to anti-collagenase activity.

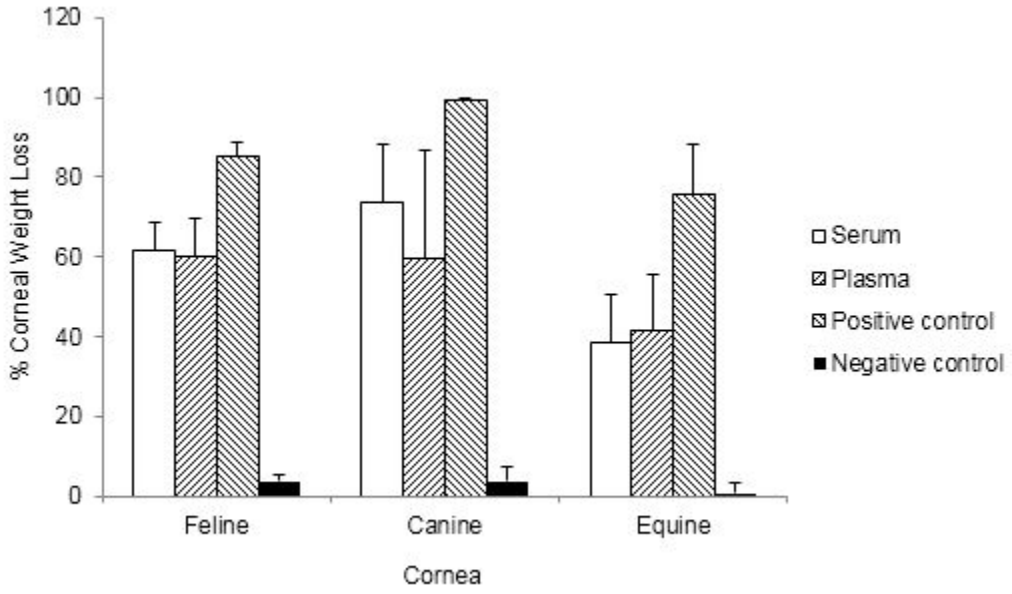

Figure 4.1 Mean (SD) percent corneal weight loss of feline, canine and equine corneal samples incubated in serum or plasma. There was no significant difference between feline (P=0.579), canine (P=0.249) or equine (P=0.406) corneas incubated with serum compared to plasma.

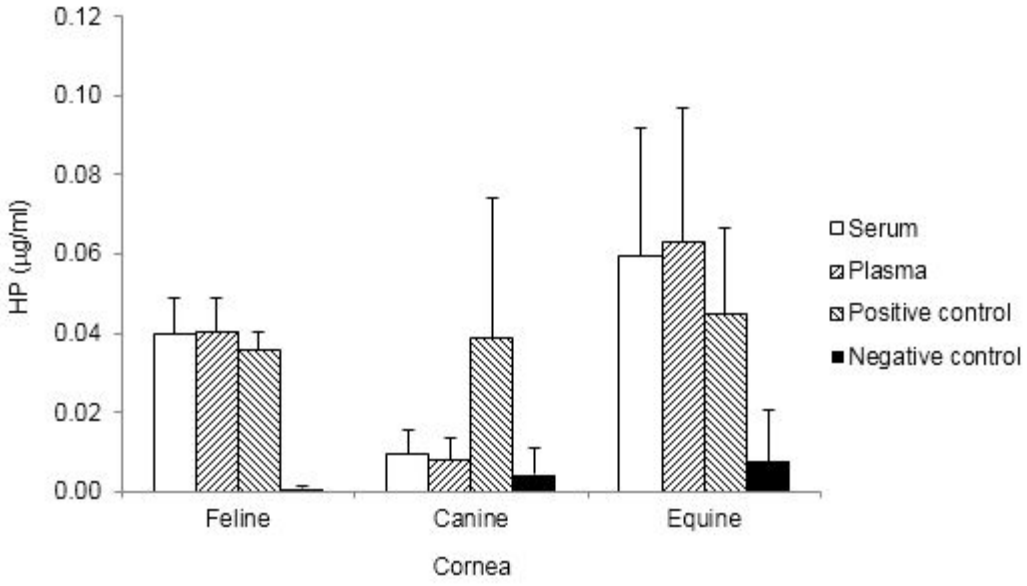

Figure 4.2 Mean (SD) HP levels of feline, canine and equine corneal samples incubated in serum or plasma. The inclusion of canine serum (*P*=0.028) or canine plasma (*P*=0.009) significantly reduced HP concentration compared to positive controls. There was no significant difference in HP concentration between corneas incubated with canine serum or canine plasma (*P*=0.497). No significant difference in HP concentrations between positive control samples and samples incubated with feline serum or plasma (*P=0.480*) or equine serum or plasma (*P=0.846*) was found.

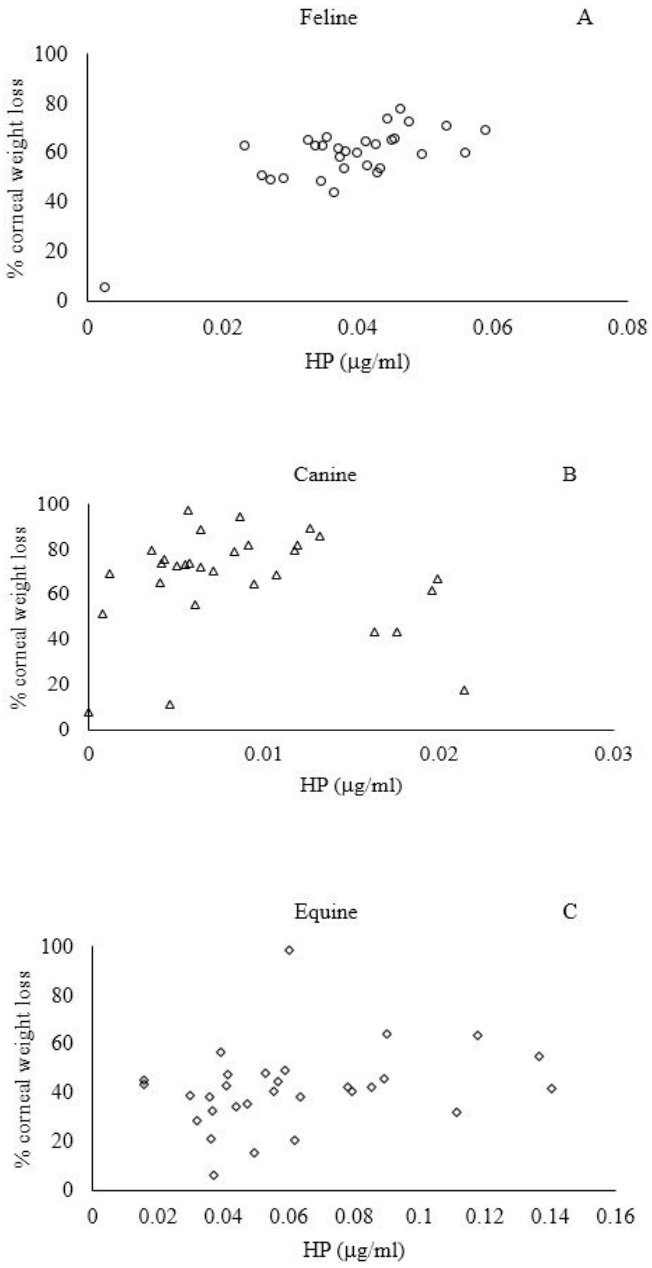

Figure 4.3 (A-C) - Scatter plots of hydroxyproline (HP) concentration versus percent corneal weight loss. A: Feline, moderate correlation; Spearman's correlation coefficient (ρ) = 0.54 (P=0.002); B: Canine, no correlation; Spearman's ρ = 0.04 (P=0.842); C: Equine, moderate correlation; Spearman's ρ = 0.31 (P=0.096)

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Footnotes:

a. BD Vacutainer serum separator blood collection tube, Becton, Dickinson and Company, Franklin Lakes, NJ.

b. BD Vacutainer lithium heparin blood collection tube, Becton, Dickinson and Company, Franklin Lakes, NJ.

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d. Whatman filter paper, Sigma Aldrich, St Louis, Mo.

e. Hydroxyproline assay kit, Sigma Aldrich, St Louis, Mo.

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CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusion

Several investigations into the anti-collagenase efficacy of blood products were undertaken over a 3-year period. The initial study evaluated the effects of storage time and storage temperature on the in vitro anti-collagenase efficacy of serum in a corneal degradation model. Secondary to this primary goal was an investigation into interspecies use of serum as an anti-collagenase. A final study aimed to compare the in vitro anti-collagenase efficacy of fresh serum to that of plasma for feline, canine and equine species. This study also examined ways to quantify corneal degradation in vitro, by measurement of percent corneal weight loss and hydroxyproline concentration in incubation media from the various experimental conditions.

Melting corneal ulcers are a frequently encountered condition in clinical ophthalmology. Regardless of the inciting cause of the ulceration, the presence of degradative enzymes from infectious agents, invading PMNL and from endogenous sources (epithelial and stromal cells) contribute to progression of stromal loss. The ability to slow or stop the destruction of the stroma can be key to saving vision or even salvage of the globe. The use of topical or systemic inhibitors of collagenases has been recommended for decades, in both human and veterinary medicine, but these

recommendations are often lacking in data to support their storage and use. As expected, addition of serum to the incubation media was protective in this in vitro model; resulting in less percent corneal weight loss compared to corneas incubated only with clostridial collagenase. This study has demonstrated that feline, canine and equine serum can be stored for up to 180 days at either -20° C or -80° C with no loss of anti-collagenase efficacy in this in vitro model. Differences in the interspecies anti-collagenase efficacy were noted. Feline serum was the most efficacious in this particular study but further research into differences in feline, canine and equine α-2 macroglobulins is needed; it is possible that amounts of α-2 macroglobulin differ between species or that they behave differently with respect to collagenase binding .

The second study demonstrated that the addition of serum or plasma was effective at decreasing the amount of corneal weight loss, compared to positive controls incubated with clostridial collagenase only. There was no significant difference in the amount of corneal weight loss that occurred for feline, canine and equine corneas incubated with serum compared to plasma. The results found here are roughly in agreement with previous studies, which found that canine plasma possessed similar anti-collagenase efficacy to that of canine serum.[1,2]

With regards to the assays of HP levels, slightly discordant results were found between the two studies performed as part of this thesis. In this first research investigation the level of HP was found to be only weakly correlated to percent corneal weight loss, even though an increase in percent corneal weight loss corresponded to

higher levels of HP. In the second study performed, the inclusion of canine serum or plasma resulted in significantly lower levels of HP while the inclusion of feline and equine serum and plasma did not significantly reduce HP levels. Overall, when comparing the HP levels of incubation fluid for feline, canine and equine corneas, there was no significant difference in the HP levels with respect to the addition of serum compared to plasma. In contrast to the first study, HP levels were moderately correlated with percent corneal weight loss for feline and equine samples but were not correlated with percent corneal weight loss for canine samples. A previous study showed correlation between corneal weight loss due to collagen degradation and HP levels of the incubation fluid.[3] The current results, along with our previous results, show that HP levels may vary widely, making their interpretation more difficult.

Current recommendations are that serum for topical use can be stored for at least 6 months at temperatures of -20°C or -80°C.

5.2 Future Directions

Future investigations in this field should include an assessment of the sterility of serum or plasma stored at varying temperatures for varying lengths of time, as this was not investigated here. As always, the results of experiments performed in a lab cannot necessarily be translated to clinical practice. An in vivo investigation into interspecies use of serum, where heterologous serum would be used topically for veterinary patients with keratomalacia, could help to determine if serum from a particular species is truly superior. An in vivo investigation into the topical utility of serum compared to that of

plasma should be undertaken, prior to the recommendation of plasma as a topical anti-collagenase for clinical patients. Also, investigations into quantifying α-2 macroglobulin levels of serum and plasma products could be performed.

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